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Decitabine-Induced Demethylation of 5′ CpG Island in *GADD45A* Leads to Apoptosis in Osteosarcoma Cells[1]

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Abstract

GADD45 genes are epigenetically inactivated in various types of cancer and tumor cell lines. To date, defects of the *GADD45* gene family have not been implicated in osteosarcoma (OS) oncogenesis, and the role of this pathway in regulating apoptosis in this tumor is unknown. The therapeutic potential of Gadd45 in OS emerged when our previous studies showed that *GADD45A* was reexpressed by treatment with the demethylation drug decitabine. In this study, we analyze the OS cell lines MG63 and U2OS and show that on treatment with decitabine, a significant loss of DNA methylation of *GADD45A* was associated with elevated expression and induction of apoptosis. *In vivo* affects of decitabine treatment in mice showed that untreated control xenografts exhibited low nuclear staining for Gadd45a protein, whereas the nuclei from xenografts in decitabine-treated mice exhibited increased amounts of protein and elevated apoptosis. To show the specificity of this gene for decitabine-induced apoptosis in OS, *GADD45A* mRNAs were disrupted using short interference RNA, and the ability of the drug to induce apoptosis was reduced. Understanding the role of demethylation of *GADD45A* in reexpression of this pathway and restoration of apoptotic control is important for understanding OS oncogenesis and for more targeted therapeutic approaches.

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Introduction

Methylation-mediated silencing of genes is one of the most important epigenetic mechanisms implicated in the regulation of normal gene expression. Such changes often affect 5′ regulatory CpG genomic regions and can be associated with aberrant expression of certain genes in cancer (reviewed in the study of Esteller [1]). Epigenetic alterations are considered to contribute in several ways to oncogenesis; for example, by activating oncogenes, by silencing tumor suppressor genes, or by disrupting pathways that contribute to tumorigenesis such as those governing apoptosis [2,3].

There is increasing interest in the use of new epigenetic therapies that might modulate molecular pathways central to tumorigenesis [4,5]. The most frequently used treatment at present is the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (decitabine) [6]. This drug is a cytosine analog that inhibits DNA methylation and can reactivate the expression of transcriptionally silenced genes. Such repression of gene expression in tumors is thought to occur by specific CpG methylation of dinucleotide clusters within CpG islands

Abbreviations: ctRNA, control nontargeting RNA; Decitabine, 5-aza-2-deoxycytidine; OS, osteosarcoma; Pyro-Q-CpG, quantitative methylation pyrosequencing; siRNA, short interference RNA

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that are present in the promoters and span exonic and intronic regions at many loci throughout the human genome [7]. Demethylation may remove tumor-specific repression within regulatory regions and permit activation of genes controlling pathways highly pertinent to oncogenesis, such as apoptosis, proliferation, invasion, and so on.

Previously, we showed that decitabine treatment of the osteosarcoma (OS) cell line U2OS led to the upregulation of >50 genes possessing CpG islands at their 5′ region [8]. One of the decitabine-activated genes of importance in OS oncogenesis was *GADD45A*. This gene belongs to the stress-responsive *GADD45* family that was reported to be methylated in multiple tumors [9,10]. *GADD45A* is relevant to OS tumorigenesis because of its central role in apoptosis and the *P53* pathway [11–13]. Moreover, Gadd45a is a central player in the maintenance of genomic stability, and loss of protein function can lead to centrosome amplification, chromosomal instability, and increased aneuploidy [14,15]. Because generalized loss of genome stability is characteristic of OS tumors [16–19], the role of this protein in OS is highly relevant. At the molecular level, the promoter region of *GADD45A* has a repression-binding site for c-MYC gene [20] that is known to be amplified in OS. Also, *GADD45A* promoter has a binding locus for *P53* in the third intronic region of the gene, and its functionality is linked to the activation of G_1/S cell cycle arrest in response to ionizing radiation [21]. Significantly, Gadd45a activation was previously shown to result in the induction of apoptosis in several cancer cell lines including COS, PC-3, DU145, and HeLa cell lines [10,22–24].

Methylation within the 5′ region of *GADD45A* is likely to be a major mechanism of repression and inactivation of the protein's apoptotic function. There is a region with dense repetitive CG sequence (CpG island) near the transcription start site (TSS) of *GADD45A* that spans 1357 bp and covers the first three exons of the gene [8,25]. Methylation of this CpG island was reported in breast cancer tissues [25]. A cluster of eight CpG dinucleotides within the first intron of *GADD45A* was found to be methylated in the OS cell line U2OS *in vitro* and in xenografts [8]. Our previous study demonstrated that induction of apoptosis in U2OS followed decitabine treatment, but the precise role of *GADD45A* CpG island demethylation and the specificity of Gadd45a expression in the induction of apoptosis were not defined. The present study was designed to examine the role of decitabine-dependent *GADD45A* CpG island demethylation on the expression of the gene and on subsequent induction of apoptosis in OS cell lines. These analyses will help determine whether *GADD45A* is a potential therapeutic effector and useful biomarker of apoptosis for future clinical trials in OS that involve genome-wide demethylation.

Materials and Methods

Cell Cultures and Treatment

The human OS cell lines U2OS (ATCC # HTB-96) and MG63 (ATCC # CRL-1427) and cervical adenocarcinoma (HeLa) cell lines (ATCC # CCL-2) were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained in ATCC recommended medium. Normal osteoblasts are a primary osteoblasts from the hipbone of normal male donor that were purchased from PromoCell, Heidelberg, Germany (catalogue # C-12760) and maintained in medium provided by the manufacturer and used at culture passage 3. Treatment with decitabine was performed as described by Liang et al. [26]. Briefly, 5×10^5 cells were plated in 56-cm^2 culture plates with 10 ml of growth medium. At 12 hours after plating, they were treated with freshly prepared decitabine (Sigma Chemical Co., St Louis, MO) to a final concentration of 1 μM without changing the medium. Decitabine was added to the medium only once to minimize the drug toxicity that is not resulting from drug effects on DNA methylation [26]. Three days after initiating the treatment, cells were harvested for RNA and DNA extraction or Hoechst 33342 staining. The use of a single-dose decitabine to significantly reactivate methylation-silenced genes was reported in bladder cancer cells [26]. Other treatment schedules analyzing demethylation in lung, head, and neck tumors have involved fresh decitabine when culture media is replaced [27].

U2OS Xenografts and Treatment

Decitabine treatment of U2OS xenografts was previously described [8]. Immune-deficient mice were bred and maintained, and xenograft experiments were performed by the Animal Resource Centre at the British Colombia Cancer agency, Vancouver, Canada. Xenografts were established under the renal capsule, and host mice were treated intraperitoneally with three doses (2-day intervals) of decitabine (2.5 mg/kg body weight) dissolved in saline (0.9% w/v NaCl) or saline alone (control). Mice were sacrificed 5 days after the last treatment, and the xenograft tissues were snap-frozen or were prepared in paraffin and sectioned using standard procedures [28].

Knockdown By Gadd45a-siRNA and Transient Transfection of GADD45A

Silencer predesigned short interference RNA (siRNA) for human *GADD45A* (siRNA ID: 146174) and control nontargeting RNA (ctRNA) were obtained from Ambion, Foster City, CA. Transfection of OS cells with siRNA was performed using siPORT Amine transfection agent (Ambion) in six-well plates according to manufacturer's protocol at the concentration of 60 nM. Decitabine (1 μM) treatments were done 12 hours after plating. RNA and protein was extracted at specified time points. For the transient transfection experiments, 2 μg of pCMV(*GADD45A*) or the empty pCMV vectors (TrueClone; OriGene, Rockville, MD) with 10 μl of Lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada) were used in the transfection experiments in six-well plates according to the manufacturer's protocol. Three days later, protein was extracted for Western blot analysis.

Quantitative Bisulfite Pyrosequencing (Pyro-Q-CpG)

Pyro-Q-CpG primers and protocol were previously described [8]. Genomic DNA from the control and decitabine treatment was bisulfite-treated using the Zymo DNA Methylation Kit (Zymo Research, Orange, CA). Bisulfite-treated DNA was PCR-amplified and sequenced according to standard protocol (Biotage, Kungsgatan, Sweden). The criteria for a CpG island was based on those outlined by Takai and Jones [29], also see [8], where the GC ≥ 55%, Obs/Exp ≥ 0.65, and length >300 bp which was reported to exclude most *Alu*-repetitive elements. We identified the genes that harbored CpG island within a 2000-bp window upstream or downstream from the TSS–based Human Genome Browser database (http://genome.ucsc.edu/). To be certain that there were no CpG island closer to the TSS and gene promoter regions, we submitted the sequences of interest (including a 2000-bp window upstream and downstream from TSS) to the CpG search engine available in Reference [29].

Reverse Transcription and Quantitative Real-Time PCR

Total RNA from U2OS, MG63, and NHOst cells was extracted using TRIzol method (Invitrogen, Osaka, Japan). Total RNA was reverse-transcribed with the GeneAmp kit (Applied Biosystems; ABI, Foster City, CA). About 20 ng of the resulting cDNA was used for *Taq*Man real-time PCR *GADD45A* expression assay (Hs00169255_m1; ABI). All reactions were done in triplicate in a 384-well plate using the 7900 Sequence Detector System (ABI). Data analysis was performed by applying the $\Delta\Delta C_t$ method relative to nontreated osteoblast with β-actin as reference gene.

Immunohistochemistry and Image Analysis

Xenograft tissue and pelleted paraffin-embedded formalin-fixed HeLa cell sections were deparaffinized and rehydrated before incubation at room temperature in 3% H_2O_2 in PBS for 10 minutes. Slides were washed in PBS three times for 3 minutes each. Antigen retrieval

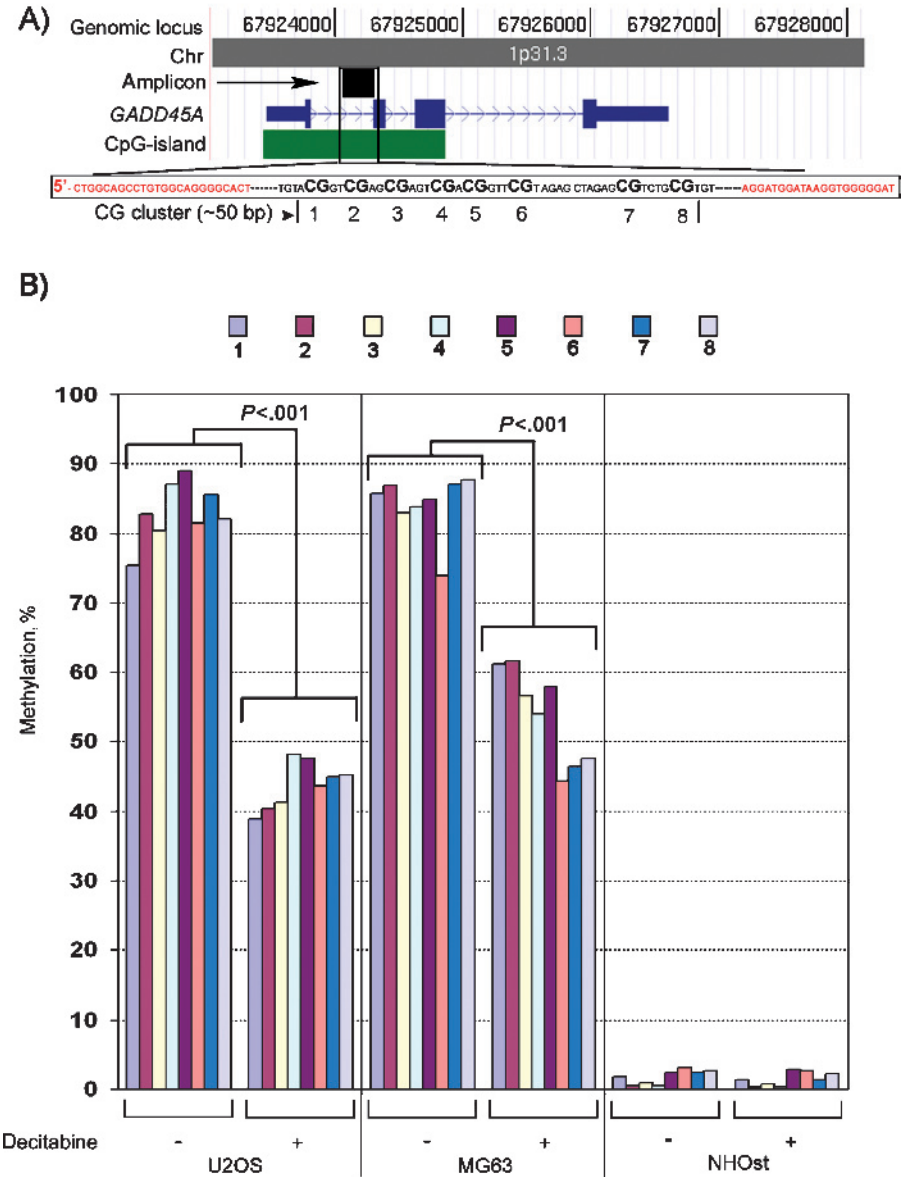

Figure 1. *GADD45A* CpG methylation in osteosarcoma cells. (A) Schematic of GADD45A genomic locus. 5′ CpG island spanning the first three exonic regions of *GADD45A* is shown. An amplicon of ~250 bp was amplified, and a cluster of eight CG dinucleotides in a ~50-bp region within the amplicon was analyzed by Pyro-Q-CpG. The schematic was taken from the latest build of the Human Genome Browser database: http://genome.ucsc.edu/cgi-bin/hgGateway. The CpG island conforms to the criteria detailed in the Materials and Methods section. (B) Percentage of methylated alleles on the eight CpG positions in U2OS, MG63, and osteoblasts as detected by Pyro-Q-CpG. Each data column is the mean percentage of methylated alleles for each CpG position in three experiments. The *y*-axis indicates the percentage of methylation, and the samples are indicated on the *x*-axis. The location of CpG positions relative to the gene start site and to each other was shown previously [8]. Methylation percentage in U2OS, MG63, and normal osteoblasts (NHOst) with (+) and without (−) 1 μM decitabine is shown for eight CpG positions in *GADD45A* promoter.

was performed at 95°C for 30 minutes in 10-mM sodium citrate (pH 6.0). After cooling in a running water bath for 5 minutes and PBS wash, the slides were blocked in 3% skim milk for 30 minutes. Blocking solution was rinsed briefly, and slides were incubated at 4°C overnight with 500× dilution of primary anti–human Gadd45a-Ab (Abnova, Taipei City, Taiwan). After the PBS wash, subsequent steps were performed using DakoCytomation Kit (Dako, Glostrup, Denmark) as per manufacturer's protocol. Hematoxylin was used for counterstaining, and slides were mounted and scanned by ScanScope CS (Aperio Technologies, Vista, CA) as previously described [8]. Positive controls were generated by exposing HeLa cells to ultraviolet (UV) radiation at 10 μJ/cm^2 [24]. These cells were then mounted on slides and processed identically to the experimental xenografts. The extent of positivity was determined by comparing the xenograft tissue sections to the control slides derived from HeLa cells. To confirm that each section retained immunoreactivity, a positive finding for the Ki67 antibody stain indicated that adjacent xenograft sections were positive and informative.

Western Blot Analysis

Cells were lysed in culture plates on ice using radioimmunoprecipitation buffer. Lysates were sonicated briefly on ice and centrifuged at high speed for 15 minutes at 4°C. Total protein was quantitated using Bradford reagent (Bio-Rad, Hercules, CA). About 50 μg of total protein/well was separated on acrylamide gel and was transferred to polyvinyl derivative membranes at 25 V at 4°C overnight. Membranes were blocked with 5% BSA for 8 hours at 4°C, followed by a 16-hour primary antibody incubation [1:12,000 mouse monoclonal anti–human Gadd45a-Ab (Abnova) or 1:24,000 rabbit polyclonal anti–human β-actin–Ab (Abcam, Cambridge, MA)]. The blots were washed with TBS–Tween 20 five times for 10 minutes each and a final wash with TBS. The blots were incubated with perioxidase-labeled secondary antibody (ECL-Plus kit; Amersham, Buckinghamshire, UK) as per manufacturer's instructions at a dilution of 1:20,000 and were scanned on the Typhoon 9410 scanner (Amersham) at normal sensitivity, 600 laser, and 200-μm resolution. Quantitation was performed using ImageJ software on blots from three replicates, and significance was estimated using *t* test at $P < .05$ and β-actin levels served as a control.

Hoechst 33342 Staining for Apoptotic Nuclei

The cells were trypsinized and resuspended in 1 ml of PBS. Staining was performed in 5 ml of 4% formalin solution with Hoechst 33342 dye (0.01-μg/μl final concentration). Aliquots of the samples were bar-coded before spreading the cells on glass slides. Hoechst-stained cells were blindly analyzed using a fluorescence microscope. One hundred cells were scored per slide, and the percentage of stained (apoptotic nuclei) out of total was recorded. Triplicate experiments were analyzed using Student's *t* test at $P < .05$.

Statistical Test

Student's *t* test was applied to analyze the differences between treatment groups.

Results

CpG Methylation of GADD45A in OS Cells

GADD45A repression of expression in U2OS *in vitro* and in xenografts was shown previously by our group to be associated with 5′

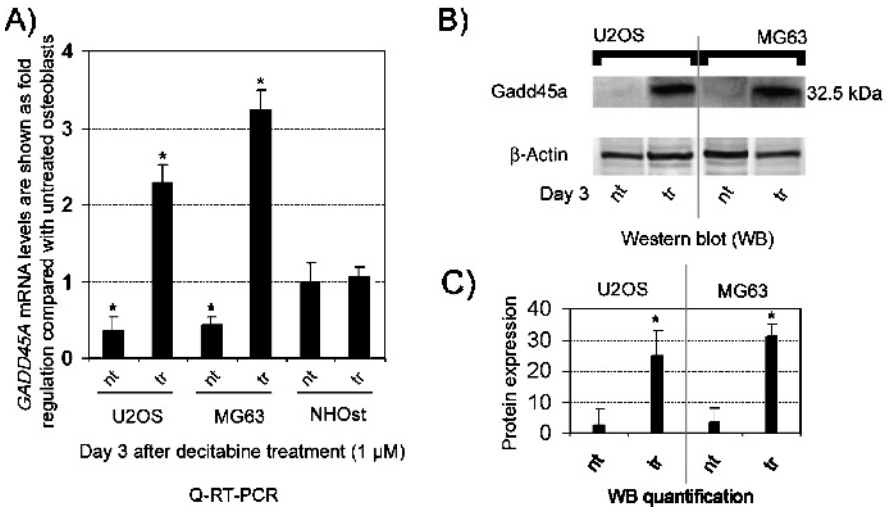

Figure 2. Repression of GADD45A expression. (A) Induction of *GADD45A* mRNA expression in U2OS and MG63 after decitabine treatment. Total RNA was extracted on day 3 after decitabine treatment was initiated. cDNA was then made as detailed in the Materials and Methods section. *Taq*Man assays were used to determine relative expression using the cDNA from untreated osteoblast as baseline and β-actin for a reference gene by applying the $\Delta\Delta C_t$ method. Each column is the mean of three replicates, and error bars indicate standard deviation from the mean. In these analyses, *GADD45A* mRNA levels are shown as fold regulation compared with the untreated normal osteoblasts. *NHOst* indicates normal human osteoblasts; *nt*, no treatment; *tr*, treated by decitabine. (B and C) Decitabine-related Gadd45a protein induction in U2OS and MG63 cells. Protein preparations from cells with or without treatment were done 3 days after initiating the treatment. For each sample, a total of 50 μg of protein was examined by Western blot analysis, and β-actin was used as a loading control. The shown blot in (B) is representative from three experiments, and the mean ± SD from quantification of three replicas after normalization to β-actin is shown on the graph in (C). Asterisks denote statistical significance compared with the no-treatment cells and nontreated osteoblasts.

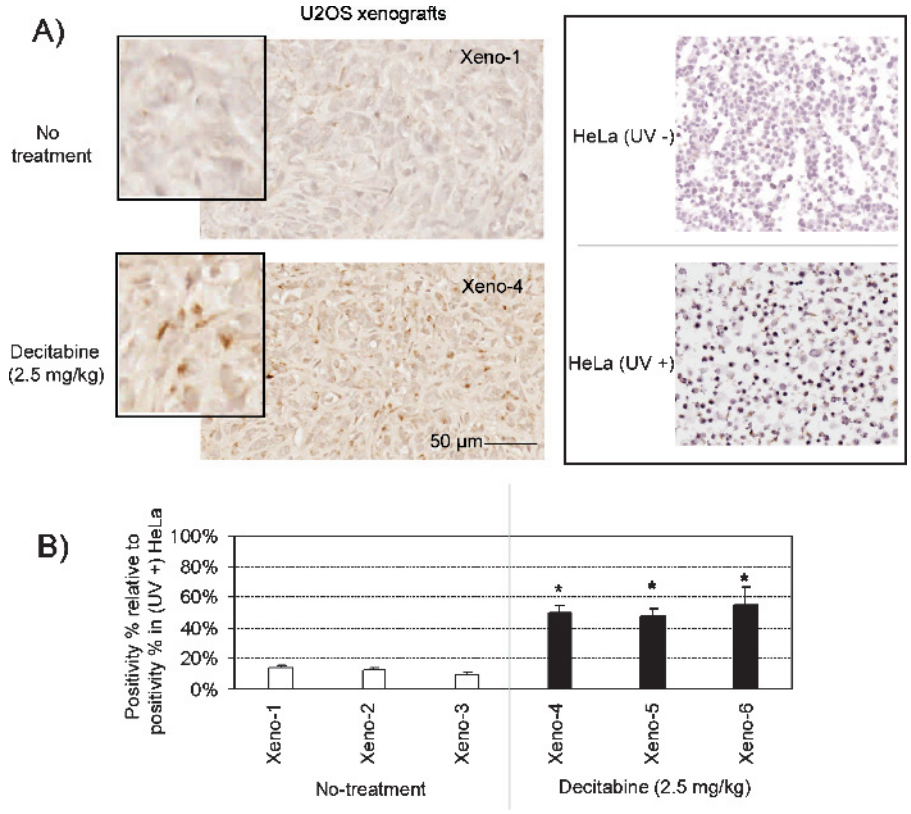

Figure 3. Induction of Gadd45a protein in OS xenografts by decitabine treatment. (A) Induction of Gadd45a protein levels in U2OS xenografts. Analysis of the relative levels of nuclear Gadd45a protein within xenografts derived from representative control untreated mice (upper panel) or decitabine-treated mice (lower panel) using immunohistochemical staining with human Gadd45a-Ab. A ×20 enlargement of representative U2OS histology is shown inside the boxed image. To the right (lower image) in (A) is the darkly stained HeLa cells corresponding to the induction of Gadd45a after UV exposure (UV+) which was used as positive control. (B) Quantification of Gadd45a-antibody staining. The staining intensity from control (light columns) and decitabine-treated (dark columns) was quantitated using Aperio scanning image analysis of sections. Gadd45a-Ab intensity was calculated based on the percentage of positivity (total positivity/total negativity per snapshot) relative to the positivity index in the positive control (UV-irradiated HeLa cells) after normalization to negative controls (no Gadd45a-Ab). Determination of the difference between the control and treated xenografts was done by applying Student's *t* test, and $P < .05$ was considered significant. The columns are the means ± SDs from 5 to 10 (~0.3 mm^2) images after normalization to positive control. The asterisk denotes statistical significance compared with the no-treatment xenografts.

CpG island hypermethylation of a cluster of eight CpG dinucleotides, within the first intronic region of the gene 620 bp downstream of the TSS (Figure 1*A*) [8]. In this study, quantitative bisulfite pyrosequencing (Pyro-Q-CpG) showed that ~80% of the sequenced alleles from the eight CpG dinucleotides in *GADD45A* of MG63 were methylated (Figure 1*B*). Exposure of MG63 to 1-µM decitabine significantly decreased methylation ($P < .001$) to ~55% of sequenced alleles at the eight CpG dinucleotides (Figure 1*B*). Moreover, methylation repression of *GADD45A* in MG63 was implicated because mRNA expression was found to be reduced at levels slightly lower than those of normal low-passage osteoblasts (Figure 2*A*). Importantly, loss of DNA methylation was associated with a 3.5-fold increase in *GADD45A* mRNA expression relative to normal osteoblasts and a six-fold increase relative to untreated MG63 cells as detected by quantitative real-time PCR (Figure 2*A*). Similar observations were found in U2OS cells, which confirmed the previous results published by our group [8]. Interestingly, very low methylation (range, 0.1–2.9% methylation of sequenced alleles) was observed at all eight CpG dinucleotides in both treated and untreated osteoblasts (Figure 1*B*). Moreover, *GADD45A* mRNA expression in osteoblasts was not induced after decitabine treatment (Figure 2*A*).

To confirm that induction of mRNA expression led to a concomitant increase in Gadd45a protein levels, Western blot analyses were performed on cell extracts from both decitabine-treated and untreated U2OS and MG63 cells (Figure 2, *B* and *C*). In both cell lines, the levels of Gadd45a protein induction after decitabine treatment were similar to that observed in the mRNA levels.

Decitabine Induces Significant Levels of Gadd45a Protein in OS Cells

To investigate the level of Gadd45a protein induction in U2OS xenografts, six xenografts (three from no treatment and three from decitabine-treated) were analyzed by immunohistochemistry using a human Gadd45a antibody. Sections from cell pellets prepared from the Gadd45a-activated UV-treated HeLa cells were used as positive controls. The staining of xenograft nuclei was quantitated using the intensity of staining and the proportion of positive tumor nuclei. As shown in Figure 3, *A* and *B*, xenograft sections from the untreated

control mice exhibited low nuclear staining for Gadd45a, whereas the staining was stronger and more frequent in nuclei from xenografts in decitabine-treated mice ($P < .05$). Induction of Gadd45a was also found to be associated with increased apoptosis as shown in a previous analysis on the same U2OS xenografts [8]. In addition, caspase-9 activation was assessed by immunohistochemistry in U2OS xenografts using an antibody specific for cleaved (activated) caspase-9. In this analysis, there was no significant induction of cleaved caspase-9 ($P > .05$) as a result of decitabine treatment when compared to no-treatment xenografts.

Gadd45a-Specific Induction of Apoptosis

Decitabine treatment at 1 μM significantly induced apoptosis *in vitro* ($P < .05$) in both cell lines in comparison to no-treatment cells (Figure 4*A*). Similarly, transient transfection of Gadd45a overexpression vector significantly induced apoptosis ($P < .05$) in untreated U2OS and MG63 in comparison to transfection with an empty vector (Figure 4*A*). In both experiments, Western blot analysis demonstrated activation of the Gadd45a protein (Figure 4, *B* and *C*). To investigate the specificity of Gadd45a induction on decitabine-induced apoptosis, *GADD45A* mRNAs were disrupted using specific siRNA in U2OS and MG63 cells. The effect of 1-μM decitabine treatment on *GADD45A* mRNA and protein levels 3 days after treatment was analyzed alone or in combination with 60 nM Gadd45a-siRNAs. Transfection of Gadd45a-siRNA against a decitabine-treated background reduced *GADD45A* mRNA levels in both cell lines by >70% knockdown efficiency (Figure 5*A*). This knockdown efficiency was consistently observed in the protein level in U2OS and MG63 cells as analyzed by Western blot analysis (Figure 5, *B* and *C*). Decitabine treatment resulted in a five-fold induc-

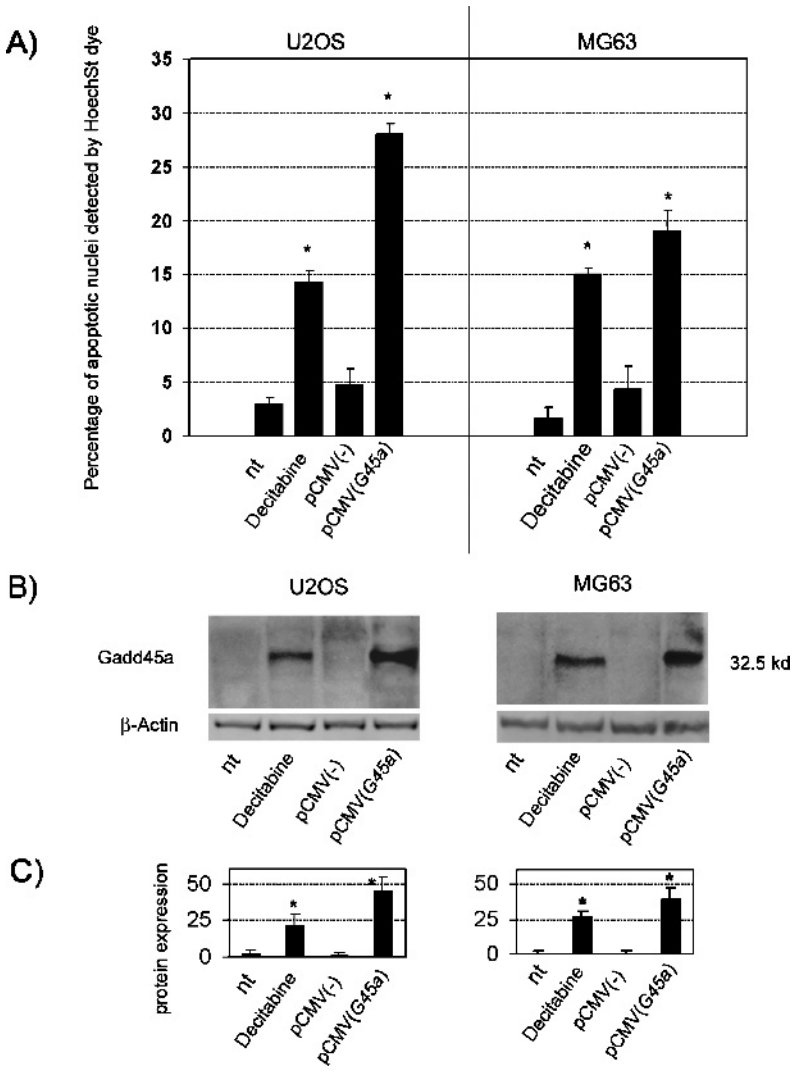

Figure 4. Apoptosis induction by pCMV-GADD45A in U2OS and MG63. (A) Percentage of apoptotic nuclei as detected by Hoechst stain in U2OS and MG63. Columns are mean of three replicas, and error bars are standard deviation from the mean. (B) Induction of Gadd45a protein by decitabine treatment or by Gadd45a transient transfection. Western blot analysis was used to detect protein levels after treatment with 1-μM decitabine or 2 μg of pCMV(*GADD45A*) transient transfection vectors (TrueClone). About 10 μl of Lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada) was used in the transient transfection experiments. (C) The mean ± SD from quantification of three replicas after normalization to β-actin. *nt* indicates no treatment; *pCMV(−)*, empty vector; *pCMV(GADD45A)*, human *GADD45A* cDNA cloned in pCMV vector. The asterisk denotes statistical significance compared with the no-treatment or empty vector.

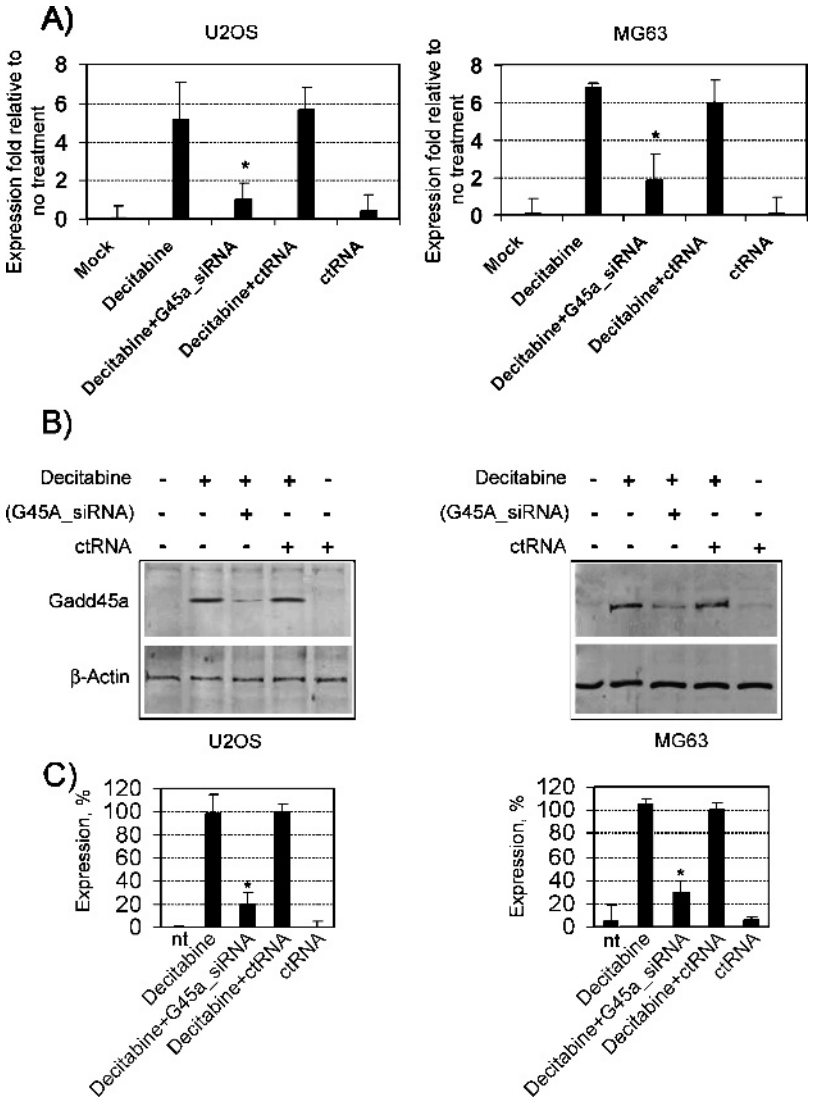

Figure 5. Reduction of GADD45A expression by *GADD45A*-siRNAs in decitabine-treated U2OS and MG63 cells. (A) Effects of GADD45A-siRNA on mRNA levels in U2OS and MG63. Total RNA was extracted on day 3 after the decitabine treatment was initiated. Preparation of cDNA and determination of mRNA relative expression is similar to that outlined in Figure 1*C* and detailed in the Materials and Methods section. Each column is the mean of three biologic replicas, and error bars indicate standard deviation from the mean. Data are expressed as fold change relative to no treatment. *Mock* indicates transfection agent + medium (no siRNA or ctRNA). (B) Gadd45a protein levels at day 3 after decitabine treatment. About 50 μg of protein was examined by Western blot analysis for the activation of Gadd45a protein, and β-actin was used as a loading control. (C) Quantification of Gadd45a protein levels. The level of Gadd45a in the decitabine-treated and scrambled control RNA-transfected (ctRNA) was set as 100%. The columns are the means ± SDs of three replicas after normalization to β-actin.

tion of *GADD45A* mRNA in U2OS, and six-fold in MG63 cells. Similar levels of induction were seen in the presence of 60 nM of scrambled ctRNA in decitabine-treated cells.

To investigate the effect of knocking down Gadd45a protein on apoptosis levels, nuclei from U2OS and MG63 were stained with Hoechst 33342 dye at day 3 after treatment in the decitabine-alone and combination experiments (Figure 6*A*). Fluorescent microscopy demonstrated that the fractions of fragmented nuclei caused by 1-μM decitabine treatment were 14% in U2OS and 15% in MG63, whereas the no-treatment cells or the ctRNA (60 nM) cells (in the presence of a transfection agent) had a fragmented nuclei (apoptotic nuclei) percentage of less than 4% in both cell lines (Figure 6*B*). Decitabine also induced apoptosis to similar levels in U2OS and MG63 when used in combination with same amounts of ctRNA and transfection agent. Interestingly, when Gadd45a-siRNA (60 nM) was used in combination with decitabine, apoptotic nuclei were reduced from 14% to 6% ($P < .05$) in U2OS and from 15% to 8% in MG63 ($P < .05$) (Figure 6, *A* and *B*), indicating that decitabine's ability to induce apoptosis was reduced specifically by Gadd45a-siRNA.

Discussion

Osteosarcoma is a particularly aggressive cancer in which current treatment modalities result in a 5-year event-free survival in 60% to 70% of patients [30,31]. The chemotherapeutic drugs most commonly used in the treatment of OS include doxorubicin, high-dose methotrexate, *cis*-platinum, and ifosfamide either alone or with

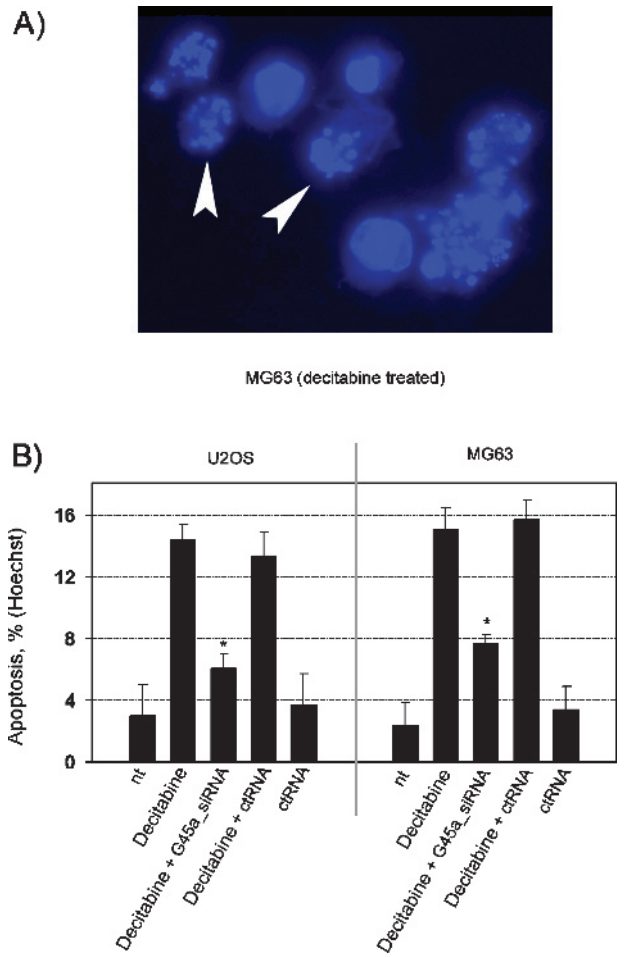

Figure 6. Gadd45a-siRNA treatment abolishes decitabine-induced apoptosis. (A) A representative image of apoptotic nuclei (arrows) from decitabine-treated MG63 stained with Hoechst 33342 dye. (B) One hundred nuclei were counted per slide, and three slides were prepared for each treatment condition. The columns are the means ± SDs of three biologic replicas. When Gadd45a-siRNA (60 nM) was added to the medium in combination with decitabine, apoptotic nuclei were reduced from 14% to 6% ($P < .05$) in U2OS and from 15% to 8% in MG63 ($P < .05$). *ctRNA* indicates control nontargeting RNA; *G45a*, Gadd45a; *nt*, no treatment; *siRNA*, short interference RNA.

etoposide (reviewed in the study of Uchida et al. [32]). Unfortunately, there is a poor response to chemotherapy in a significant subgroup of OS patients, and some patients have a high drug toxicity profiles [33]. Attempts at changing chemotherapy regimens for poor responders have generally failed to improve outcome [34].

In recent years, novel therapeutic approaches involving genome-wide epigenetic modification have been introduced (reviewed in the study of Issa [6]). Decitabine is one of the most popular in this class of new drugs, and it has been approved by the US Food and Drug Administration [35]. The results of early clinical trials using this drug are promising, but there is little detail available concerning the molecular pathways leading to tumor response (reviewed in the study of Kihslinger and Godley [36]). The mechanisms of response to decitabine may vary in different patients, but they are thought to include induction of senescence, differentiation, and apoptosis (reviewed in the study of Issa [6]).

Previously, we showed that decitabine treatment of the OS cell line U2OS led to the upregulation of >50 genes possessing CpG islands at their 5′ region [8]. *GADD45A* was one of the decitabine-activated genes that were known to be involved in apoptosis, and importantly, the function of the protein is relevant to OS oncogenesis. Interestingly, the GADD45 stress-response family has been shown to be inactivated by methylation in several types of tumors [9] (reviewed in the study of Zerbini and Libermann [10]). The protein has been implicated in regulating genome stability (reviewed in the study of Hollander and Fornace [15]), DNA damage response [11], DNA repair [37,38], apoptosis [24], and most recently, when overexpressed, DNA demethylation [39]. Despite the impact of *GADD45A* gene on all these processes, and its frequent methylation in some tumors, there is limited information regarding the role of *GADD45A* in the response to epigenetic modifications, in general, and to demethylation treatment, in particular.

In this study, we found that extensive methylation of the 5′ CpG region of *GADD45A* was present in the MG63 and U2OS cell lines, and this epigenetic change was associated with the reduced expression of *GADD45A* in OS. Furthermore, it was found that exposure of both OS cell lines to decitabine significantly decreased the methylated alleles to ~55% in this region of the gene. The relationship between loss of DNA methylation in this region, and elevated gene expression was demonstrated by a six-fold increase in both *GADD45A* mRNA and protein levels. The role of Gadd45a on cell cycle arrest is well established. The protein has been shown to play a role in G_2-M checkpoint in response to DNA damage. Gadd45a activates p53-dependent G_2-M arrest, providing a link between p53-dependent cell cycle checkpoint and DNA repair. In this regard, it is noteworthy that p53 inactivation is one of the most common aberrations observed in human OS (reviewed in Kansara and Thomas [40]).

The role of Gadd45a in apoptosis is not as well understood as is its role in cell cycle arrest. *GADD45A* overexpression in normal human fibroblasts and human cancer cells causes G_2-M arrest [25,41,42]. Gadd45a-dependent induction of apoptosis has been observed frequently in cancer cell lines [10,24], but its role in apoptosis induction in normal cells has been controversial. Gadd45a has been shown to induce cell cycle arrest in normal fibroblasts but fail to induce apoptosis [41]. Others, however, have shown Gadd45a-dependent induction of apoptosis in normal epithelial cells [43]. In the present study, we show that Gadd45a reexpression correlates with a significant increase in apoptosis in OS cells. *In vivo* effects of decitabine treatment were studied by establishing xenografts and showing that tumor sections of xenografts from untreated control mice exhibited low nuclear staining for Gadd45a protein, whereas the nuclei from xenografts in decitabine-treated mice exhibited much higher levels of Gadd45a protein. As with the *in vitro* studies, an increase in Gadd45a protein levels was associated with a significant increase in apoptosis.

To show the specificity for Gadd45a in decitabine-induced apoptosis, *GADD45A* mRNAs were disrupted using siRNA. This approach has been used previously in other cell types, and inhibition of *GADD45A* led to disruption of functions including its ability to induce apoptosis [10,44,45]. *GADD45A* activation in OS was found to be central to the decitabine-induced apoptosis, and knockdown by siRNA demonstrated the specificity of this effect. This observation, however, does not eliminate the possibility of the involvement of the other apoptotic factors and pathways in the decitabine-induced apoptosis in OS cells [8].

Osteosarcoma is known for having high levels of genomic instability with multiple chromosomal breakpoints and increased incidence in genomic aberrations [17–19,46]. Inhibition of the DNA damage response gene such as *GADD45A* by DNA hypermethylation could be one explanation on how OS cells escape apoptosis and undergo survival, despite the numerous DNA breakpoints required to generate the complex karyotypes that characterize the OS genome. Interestingly, Gadd45a has recently been shown to actively demethylate downstream target genes by promoting DNA repair [39] thus linking both processes. Finally, the findings of a potential role for *GADD45* repression by methylation in OS oncogenesis may encourage the development of novel therapeutic strategies that take advantage of improved understanding of the genomics and epigenomics of this tumor.

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